Journal of Chromatography, 327 (1985) 351–357
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 595

Note

Characterization of the subunit structure of dopamine β-hydroxylase by anion-exchange high-performance liquid chromatography

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Dopamine β-hydroxylase catalyzes the conversion of dopamine to norepinephrine in the presence of ascorbic acid[1] and copper[2] and, thus, is an essential enzyme for catecholamine neurotransmitter synthesis. The enzyme is located primarily in the adrenal medulla, where it exists in both soluble and membrane-bound forms.

Soluble dopamine β-hydroxylase has been shown to be a tetrameric glycoprotein with a molecular weight of 290000 daltons (D) (ref. 3). Based on dissociation patterns in the presence of denaturing and reducing agents, the enzyme is thought to be composed of four identical or nearly identical monomeric subunits of approximately 75000 D each, which are linked by disulfide bonds into two dimers of approximately 150000 D each. A pair of dimers then aggregates by non-covalent linkages to form the native enzyme[3]. While several groups have reported on the amino acid content, carbohydrate content, and N-terminal sequence of soluble dopamine β-hydroxylase, the results of these studies show little consistency[3]. It has been suggested that the variation in results is due to the use of non-homogenous preparations of the enzyme[3]. Previous work in this laboratory showed that purification of a crude chromaffin granule lysate by two conventional size-exclusion gel chromatography steps yielded a preparation that appeared to give a single band with Coomassie Blue staining after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[4]. However, when a more sensitive silver-staining technique was used, many contaminating bands were observed. Further, the more sensitive stain revealed that the dopamine β-hydroxylase band was, in fact, a cluster of three bands, thus suggesting that the enzyme is composed of heterogeneous monomers.

The work presented here describes our attempts to purify further the native enzyme by ion-exchange high-performance liquid chromatography (HPLC) and the implications of our results in terms of the subunit structure of the enzyme.

EXPERIMENTAL

Protein purification prior to HPLC

The purification scheme for soluble dopamine β-hydroxylase prior to the HPLC step has been previously reported[4]. Combined active fractions from the last

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0021-9673/85/$03.30

step in that scheme (*i.e.*, a Sepharose-4B column) were then subjected to further purification by HPLC.

High-performance liquid chromatography

The HPLC instrumentation consisted of a Waters Model 660 solvent programmer, Model M-45 and Model 6000A solvent delivery systems, a Model U6K injector, a Model 441 absorbance detector, fitted with a mercury lamp, and a 254-nm filter (Waters Assoc., Milford, MA, U.S.A.), and an Omniscribe strip-chart recorder (0.001–10 V, Houston Instruments, Austin, TX, U.S.A.). The anion-exchange column was a Synchropak AX-300, 100 × 4.1 mm I.D. column (Synchrom, Linden, IN, U.S.A.). A silica gel pre-column (Whatman, Clifton, NJ, U.S.A.) was placed ahead of the injector to minimize column deterioration at basic pH without affecting the protein chromatography characteristics.

For dopamine β-hydroxylase purification, a two-component buffer system was used. Component A was 20 m*M* potassium phosphate buffer (pH 6.8–7.4) and component B was the same potassium phosphate buffer as A plus 0.8 *M* sodium chloride. Chromatography was performed by equilibrating the column in 95% A (0.04 *M* sodium chloride). Up to 1 mg of Sepharose-4B-purified protein was applied in 500 μl of buffer A. Non-retained protein was eluted by washing with the equilibration buffer for 8 min at 1 ml/min. Dopamine β-hydroxylase was then eluted by a 45-min linear gradient of 95% A: 5% B (0.04 *M* sodium chloride) to 5% A: 95% B (0.76 *M* sodium chloride) at 1 ml/min. Fractions of 0.5 ml each were collected during the gradient elution.

Electrophoresis

SDS–PAGE was performed according to Laemmli[5], and proteins were visualized by a highly sensitive silver-staining procedure[6]. Relative protein concentrations were determined by densitometry (Hoeffer Instruments, San Francisco, CA, U.S.A.).

RESULTS AND DISCUSSION

Anion-exchange HPLC was chosen for further purification of the Sepharose-4B-purified preparation of dopamine β-hydroxylase because (i) DEAE-cellulose column chromatography had been successfully incorporated into other purification schemes for the enzyme[7]; and (ii) gel chromatography had already been used in the two previous steps in our purification scheme[4]. As can be seen in Fig. 1, anion-exchange chromatography yields purified dopamine β-hydroxylase. The three monomeric subunits remain but all contaminating proteins have been eliminated (the faint bands observed at a lower molecular weight than the three dopamine β-hydroxylase bands are minor contaminants in the PAGE sample buffer and are also seen in the lane with the molecular-weight standards). Anion-exchange HPLC is much faster than conventional DEAE-cellulose chromatography (53 min for a complete HPLC run compared to greater than 8 h for DEAE-cellulose[7]) and it also provides excellent purity and recovery of activity.

As the pH of the elution buffer was raised from pH 6.0 to 6.8, 7.0, and 7.4, the recovery of total units of dopamine β-hydroxylase increased from 15% to 86%, as is shown in Table I. The enzyme has previously been shown to be stable in that

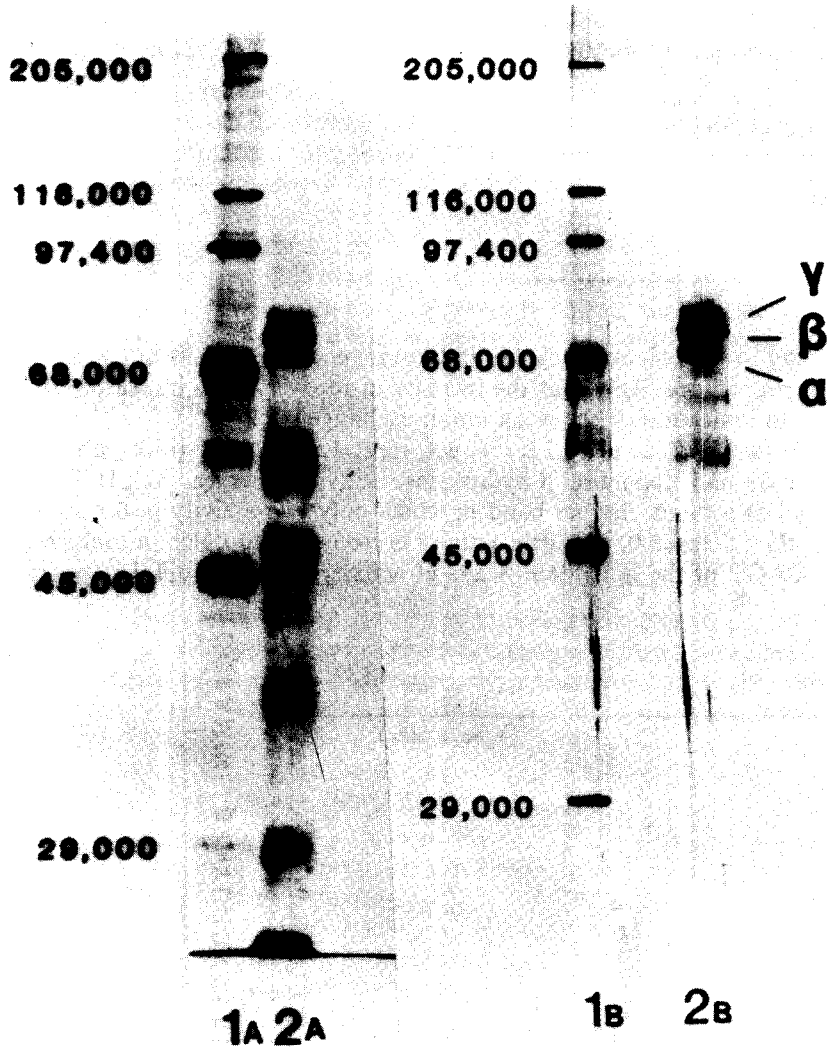

Fig. 1. SDS–PAGE of Sepharose-4B-purified dopamine β-hydroxylase preparation before and after anion-exchange HPLC. Lane 1A, molecular-weight standards; lane 2A, Sepharose-4B-purified preparation; lane 1B, molecular weight standards, lane 2B, HPLC-purified dopamine β-hydroxylase.

pH range, and all assays were performed at the same pH, so the increase represents an actual increase in recovery of protein.

The dramatic effect of pH changes in this range is interesting, since the pI of dopamine β-hydroxylase has been estimated at 4.7–5.5, based upon urea gel electrophoresis[8] and elution of dopamine β-hydroxylase activity at pH 4.7 from a HPLC chromatofocussing column[9], performed as described by Wagner and Regnier[10]. The estimated pI is consistent with the high glutamic acid content in the protein[3]. Although the enzyme is already substantially ionized at pH 6.0, one would expect an increased pH to result in a greater proportion of ionized groups on the protein,

TABLE I

EFFECT OF ELUTION pH ON RECOVERY OF DOPAMINE β-HYDROXYLASE BY ANION-EXCHANGE HPLC

pH	*% of total units recovered*
6.0	15
6.8	28
7.1	70
7.4	86

increased binding to the column packing, and lower recovery. This is not what was observed. It appears more likely that the increase in recovery with increased pH is due to changes in ionization of the weak anion-exchange packing.

As is seen in Fig. 2, at pH 7.1 a distinct, reproducible multi-peak pattern of both protein peaks and dopamine β-hydroxylase activity emerges. At pH 7.4 the enzyme is eluted as a much sharper band in which only two activity peaks are observed and at pH 6.8 and 6.0, too little activity is recovered to detect a multi-peak pattern. SDS–PAGE of the individual peaks of activity, eluted at pH 7.1 (peaks 1

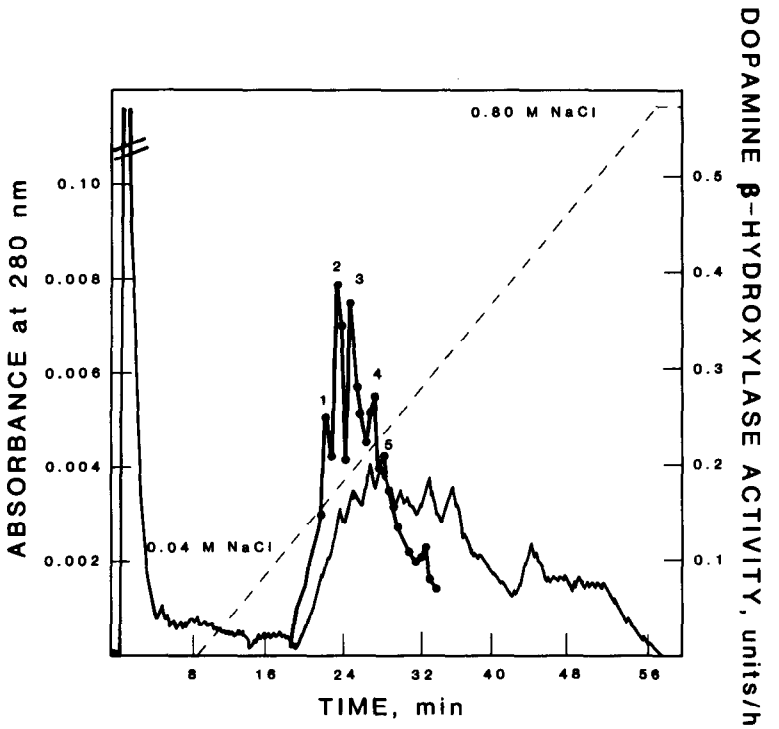

Fig. 2. Purification of dopamine β-hydroxylase by anion-exchange HPLC at pH 7.1. The Sepharose-4B-purified preparation was applied and eluted from the Synchropak AX-300 column, as described. Dopamine β-hydroxylase was eluted at a salt concentration between 0.21 and 0.33 *M* sodium chloride. Protein, determined by absorbance at 254 nm (———). Enzyme activity, ●.

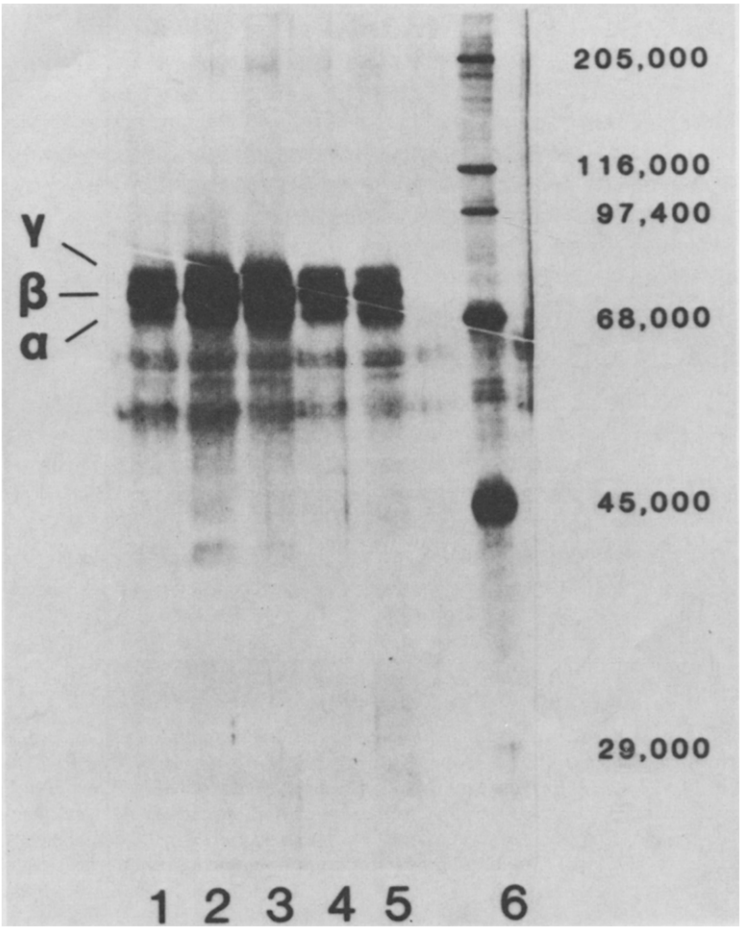

Fig. 3. SDS–PAGE of anion-exchange HPLC purified dopamine β-hydroxylase. The peaks of dopamine β-hydroxylase activity eluted at pH 7.1, as seen in Fig. 2, were analyzed individually on 10% SDS–polyacrylamide gels: lane 1, peak 1 (as labeled in Fig. 2); lane 2, peak 2; lane 3, peak 3; lane 4, peak 4; lane 5, peak 5.

through 5), followed by densitometry, revealed that the proportion of the individual monomeric subunits varied in the elution pattern, as is seen in Figs. 3 and 4. The protein corresponding to the first peak contained more γ-subunit than α-subunit, and the fifth peak contained substantially more α-subunit than γ. As can be seen in Fig. 4A, the specific activity of individual peaks varies, even though each peak appears to be homogenous dopamine β-hydroxylase. These results suggest that the native enzyme, a tetramer, exists in a variety of forms, which differ in the proportion of the individual monomeric subunits.

Attempts to purify the monomers by HPLC were unsuccessful. Enzyme which had been dissociated into monomers was subjected to HPLC under conditions similar to those which resulted in the purification of native enzyme, but with 0.25% emulphogen and/or β-mercaptoethanol or dithiothreitol included in the buffers. Unfor-

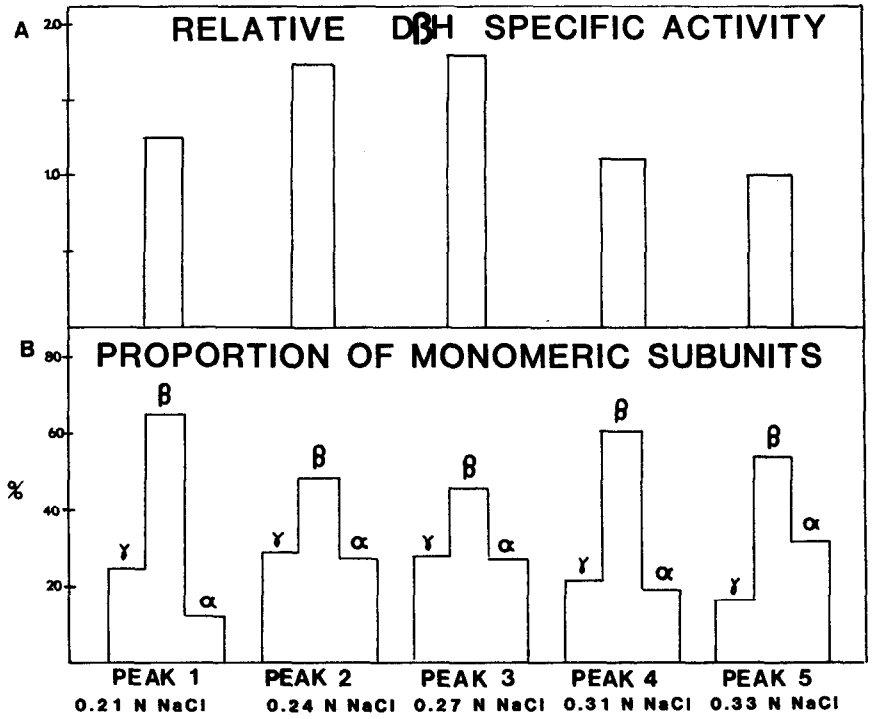

Fig. 4. Dopamine β-hydroxylase enzymatic specific activity and subunit composition, corresponding to the individual peaks obtained by elution at pH 7.1. Dopamine β-hydroxylase was purified by HPLC, as described and illustrated in Figs. 2 and 3. Enzyme activity was measured as described by Wong *et al.*[4]. Protein from each peak was separated on SDS–PAGE, as seen in Fig. 3 and the monomers were quantitated by scanning densitometry. (A) Enzyme-specific activity of each peak, expressed relative to the peak with the lowest specific activity (peak 5). (B) Amount of α,β, and γ monomer in each peak, normalized to equivalent amounts of total protein.

tunately, the limiting factor was the instability of the monomers in the presence of salt, even at low concentrations and in the presence of glycerol. However, the individual monomers could be separated by elution from 10% SDS–PAGE gels. Monomeric subunits isolated in this manner were subsequently converted by treatment with endoglycosidase F to the same 66000-D protein, suggesting that the three subunit bands are all glycosylation variants of a single 66000-D protein[11]. Apparently, the different glycosylation patterns confer sufficiently different properties to the subunits, so that when they are combined in the proportions observed in Fig. 4B, they interact with the anion-exchange packing to produce the observed multi-peak pattern.

In conclusion, anion-exchange HPLC provides a rapid means of purifying soluble dopamine β-hydroxylase to homogeneity, even when an extremely sensitive stain is used to detect contaminants. Furthermore, HPLC has revealed a complex fine-structure of the native enzyme. We believe that the carbohydrate heterogeneity is not simply an artifact of purification since the three monomer bands are observed at each stage of purification. However, studies are continuing to reveal the significance of the complex variety of tetrameric structures in which the native enzyme exists.

ACKNOWLEDGEMENTS

This work was supported by Grant MH 25998 from the National Institute of Mental Health. R.D.C. is the recipient of Research Career Development Award MH 00219 from the National Institute of Mental Health.

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